UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

KATEDRA ANALYTICKÉ CHEMIE

FARMACEUTICKÁ FAKULTA UNIVERZITY V BOLONI
ITÁLIE

KATEDRA VÝZKUMU KOVŮ, ELEKTROCHEMIE A CHEMICKÝCH VĚD

DIPLOMOVÁ PRÁCE

VÝVOJ IMUNOCHROMATOGRAFICKÉHO STANOVENÍ PRO TESTOVÁNÍ REÁLNÝCH VZORKŮ OBSAHUJÍCÍCH TNT

Lucie Kosařová

Hradec Králové 2010/2011

CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF ANALYTICAL CHEMISTRY

ALMA MATER STUDIORUM UNIVERSITY OF BOLOGNA
FACULTY OF PHARMACY

DEPARTMENT OF METAL SCIENCES, ELECTROCHEMICAL AND CHEMICAL TECHNIQUES

DIPLOMA THESIS

DEVELOPMENT OF LATERAL FLOW IMMUNOASSAY FOR TESTING REAL SAMPLES CONTAINING TNT

Lucie Kosařová

Hradec Králové 2010/2011

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

Lucie Kosařová

Acknowledgement

This thesis could originate thanks to the Erasmus programe. I would like to express my thanks to Prof. RNDr. Petr Solich, CSc., PharmDr. Petr Chocholouš, Ph.D., Prof. Stefano Girotti and Dott.ssa Elisabetta Maiolini for their help, worth advice and patience.

Poděkování

Tato práce vznikla díky programu Erasmus. Za veškerou pomoc, cenné rady a trpělivost děkuji Prof. RNDr. Petru Solichovi, CSc., PharmDr. Petru Chocholoušovi, Ph.D., Prof. Stafano Girotti a Dott.ssa Elisabetta Maiolini.

Abstract

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Analytical chemistry

Candidate: Lucie Kosařová
Supervisor: PharmDr. Petr Chocholouš, Ph.D.
Title of diploma thesis: Development of lateral flow immunoassay for testing real samples containing TNT

Obtaining results very fast is requested more often these days. This is particularly true for dangerous and potentially dangerous compounds. One of these compounds is 2,4,6-trinitrotoluene (TNT), which was tested in this research. TNT has been extensively used as a military explosive and is still one of the most widely used explosives. Its advantages, such as low manufacturing costs, safety of handling and fairly good explosive power can be tempting for abuse by terrorists. In addition, TNT is a well known pollutant because of its toxicity and low biodegradability. It can contaminate surface and ground waters, soils and sediments, thus causing environmental and health problems.

According to these facts there is a requirement to detect this nitroaromatic compound in minimal concentrations very fast and outside the laboratory. These demands could be met by the lateral flow test.

In this research lateral flow immunoassay (LFIA) based on colloidal gold nanoparticle labels was developed for detection of TNT in real samples. The samples were obtained by controlled explosion of TNT, then collected with a cotton swab wetted with methanol and their manual extraction was performed in methanol as the solvent. LFIA procedure was carried out as a dipstick test which means the strip was dipped in the well containing small amounts of immunoreagents and sample extract.

The lateral flow test provided a yes/no response within 10 minutes which could be very useful for criminalists to reveal early warning of a terrorist attack or for ecologists to prevent environmental pollution. All samples were also

analysed by Enzyme-linked immunosorbent assay with chemiluminescent detection (CL-ELISA) and the results obtained by the two methods were correlated showing a good robustness of LFIA.

Abstrakt

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra analytické chemie

Kandidát: Lucie Kosařová
Školitel: PharmDr. Petr Chocholouš, Ph.D.
Název diplomové práce: Vývoj imunochromatografického stanovení pro testování reálných vzorků obsahujících TNT

V dnešní době je stále více vyžadováno velice rychlé získání výsledků. Zejména to platí pro látky nebezpečné a potenciálně nebezpečné. Jednou z takových látek je 2,4,6-trinitrotoluen (TNT), látka, která byla testována v průběhu této práce. TNT byl značně používán jako výbušnina pro vojenské účely a je stále jednou z nejpoužívanějších výbušnin. Jeho výhody, jako nízké výrobní náklady, bezpečná manipulace a dobrá výbušnost, může vést ke zneužití této sloučeniny teroristy. TNT je navíc známým polutantem kvůli jeho toxicitě a nízké biodegradabilit. Může kontaminovat povrchovou i spodní vodu, půdu a sedimenty, a tak způsobovat problémy jak v životním prostředí, tak zdravotní problémy.

Proto je požadováno stanovit tuto nitroaromatickou sloučeninu v minimálních koncentracích, velmi rychle i mimo laboratoř. Tyto požadavky může splnit lateral flow test.

V průběhu této práce byl vyvinut imunochromatografický test (LFIA) založený na značení koloidním zlatem pro detekci TNT v reálných vzorcích. Tyto vzorky byly získány kontrolovaným výbuchem TNT, poté setřeny vatovou tyčinkou namočenou v metanolu a jejich extrakce proběhla manuelně také do metanolu. Samotný test byl proveden jako dipstick test, což znamená, že membrána byla ponořena do jamky obsahující malé množství imunoreagencií a extraktu vzorku.

Lateral flow test poskytl kvantitativní odpověď (ano/ne) během pouhých 10 minut, což může být velice prospěšné pro kriminalisty, kteří tak můžou brzy

odhalit hrozbu možného teroristického útoku, nebo pro ekology, aby zabránili znečištění životního prostředí. Všechny vzorky testované pomocí lateral flow byly testované i pomocí enzymové imunoanalýzy s chemiluminescenční detekcí (CL-ELISA). Výsledky získané oběma metodami spolu dobře korelovaly, což dává imunochromatografickému stanovení (LFIA) dobrou robustnost.

Index

List of pictures

List of tables

Abbreviations:

BSA	Bovine Serum Albumin
ELISA	Enzyme-Linked Immunosorbent Assay
GAR	Goat anti rabbit
KLH	Keyhole Limpet Hemocyanin
LDD	Lowest detectable dose
LF	Lateral Flow
LFIA	Lateral Flow Immunoassay
LOD	Limit of detection
MeOH	Methanol
NALFIA	Nucleic Acid Lateral Flow Immunoassay
NALF	Nucleic Acid Lateral Flow Assay
OVA	Ovalbumin
PBS	Phosphate buffered saline
RAM	Rabbit anti mouse
RAM-CG	Rabbit anti mouse colloidal gold
TNBS-OVA	Trinitrobenzenesulfonate conjugated with ovalbumin
TNT	2,4,6-Trinitrotoluene

1. Objectives

The main goal of this research was to develop an economic and rapid but still reliable test for TNT detection in real samples, also outside the laboratory. These demands on testing are imposed according to the TNT properties, such as explosiveness and toxicity. For this purpose a lateral flow (LF) test was developed giving the information whether a sample contains TNT or not, knowing the limit of detection i.e. the lowest quantity of the substance that can be distinguished from the absence of that substance. The results are evaluated visually and lateral flow immunoassay (LFIA) does not require laboratory instruments, thus giving this assay further advantage to use it outside the laboratory. After previous development of the LF test for standard solution of TNT the goal was to also verify the correct functioning of this test on real samples.

2.Theoretical part

2.1 2,4,6-Trinitrotoluene

2.1.1 Structure and properties

TNT belongs to the nitro-aromatic explosive compounds [1 - 3]. It consists of a benzene ring with nitro groups on positions 2, 4, 6 and a methyl group on position 1 (**Figure 1**) [4]. This yellow colour compound does not occur naturally [2, 3, 5, 6]. It is synthesized from toluene by a nitration process [1, 4, 7], which is relatively simple in all-glass apparatus [7]. On the other hand the manufacturing process is difficult because of the requirement to use nitric acid in relatively high temperatures in the presence of hydrogen sulphate with excess of sulphur trioxide dissolved in it [7], which is used to remove undesirable non-symmetrical isomers (2,4 dinitrotoluene, 2,6-dinitrotoluene, 3,4-dinitrotoluene, 2-nitrotoluene, 3-nitrotoluene and 4-nitrotoluene) formed during synthesis of TNT [1, 4]. The final product contains few percent of impurities [4].

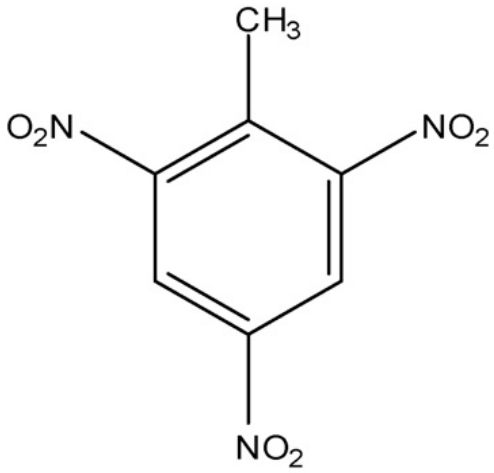

Figure 1 2,4,6-trinitrotoluene

Nitro groups of TNT are easily reducible, because both oxygen and nitrogen are two highly electronegative elements, but oxygen possesses higher electro negativity than nitrogen. The result is polarized N–O bonds within the nitro groups, which make them easily reducible [4].

The TNT molecule has an intermediate character between hydrophobic and hydrophilic properties, according to its octanol–water partition coefficient (log K_{OW} = 1.6), which designates its mobility in the environment [4]. The value

of TNT solubility in water (100 mg L^{-1} at 25℃) influences its occurrence in streams and groundwater. It is important keeping in mind that TNT solubility increases with increasing temperature and decreases with increasing pH [8]. Because it has low vapour pressure, it can be considered as absent from the atmosphere, which accounts for non-detection in atmosphere monitoring studies [4].

TNT has a yellow colour and it darkens in sunlight. This low hygroscopic compound has a crystal density of 1.654 g cm^{-3} at 20℃ and it is unstable in alkalis and amines [5]. Other physicochemical properties of TNT are mentioned in **Table 1**.

CAS number	118-96-7
Chemical formula	$C_7H_5N_3O_6$
Molecular weight	227.13
Melting point	80.1℃
Boiling point	240℃
Water solubility at 20℃	130 mg L^{-1}
Solubility in acetonitrile at 20℃	100 g/100 g
Solubility in acetone at 20℃	109 g/109 g
Vapour pressure	1.99×10^{-4} mm Hg
log K_{OW}	1.6
log K_{OC}	3.2
Henry's law constant (20 ℃)	4.57×10^{-7} atm-m^3/mol

Table 1 Physicochemical properties of TNT [4]

2.1.2 The history of TNT development

TNT was first manufactured in 1863 from toluene in Germany by Joseph Wilbrand, who developed TNT as a yellow dye [2, 5 7]. Discovery of the isomer 2,4,5-trinitrotoluene led to the first detailed study of the preparation of 2,4,6-trinitrotoluene in 1870 by Beilstein and Kuhlberh. Pure 2,4,6-trinitrotoluene was prepared in 1880 by Hepp and its structure was established in 1883 by Claus and Becker [5]. The manufacture of TNT in industrial scale as an explosive

began in Germany in 1891 and in 1899 aluminium was mixed with TNT to produce an explosive composition called aminal, which has been used as an underwater explosive [5, 7]. In 1902, TNT was adopted for use by the German Army replacing picric acid (2,4,6-trinitrophenol) and in the same year Germany began loading artillery shells with it [2, 5, 7]. In 1912 the US Army also started to use TNT. By 1914 TNT became the most widely used military explosive for armies during World War I [2, 4, 5, 9] and the highest production of it was achieved at the end of World War II when about 150 k ton were produced per month [1].

2.1.3 TNT as an explosive

The definition that an explosive is a substance used or manufactured with an intention to produce a practical effect by explosion was defined by law in the United Kingdom prior to 1983. TNT fits this definition [7] and is considered as the 'standard' high explosive to which all other high explosives have been and continue being measured [2]. High explosives are divided into primary (initiating) and secondary explosives, which are further divided into industrial and military explosives [7]. TNT belongs to military explosives [2, 7] with a detonation velocity greater than 22,000 feet per second [2]. It is considered an insensitive explosive meaning that it has low sensitivity to detonation by impact or friction, which allows TNT to be handled by workers in a relatively safe manner [2, 5, 34]. On the other hand it is necessary to prevent fugitive electricity, static electricity, or certain radiofrequencies that could lead to detonation. TNT will burn in the presence of oxygen but will explode in an oxygen-deficient environment [2]. At firstly the German army used TNT for this purpose as filling for its artillery shell at the beginning of the 20th century. During the bomb preparation TNT is melted and then poured into shell cases where it will crystallize and contract by about 10% of its volume [7]. Another way to load TNT into shells is by pressing it [5]. It can be used on its own or mixed with other components. Amatols is a mixture of TNT with ammonium nitrate [5, 7], cyclonide and composition B are mixtures of TNT and other explosive RDX (cyclotrimethylenetrinitramine) [5]. As TNT is the main compound of the mixtures used in the explosive industry, its isomers

and impurities, exudations of the isomers of dinitroroluenes and trinitrotoluenes can occur. According to this fact, especially in higher temperatures, TNT can form cracks and cavities which can lead to a reduction in density of TNT and premature detonation [5].

TNT has been the most widely used explosive for military applications due to a number of advantages [2, 4, 5, 9]. Among them we can highlight low manufacturing costs [5, 10, 34] (TNT has been manufactured from coal tar products) [5, 7], high explosive power and safety of handling [5, 10, 34]. TNT also has a low melting point for casting, good chemical stability and good compatibility with other explosives. Recently TNT is the most important explosive for blasting charges. It is also widely used in commercial explosives but only in a lower grade of TNT than has been used for military purposes [5].

2.1.4 TNT as an environmental pollutant

As mentioned above, TNT has been extensively used as a military explosive for more than 50 years, especially during World War I and II [4, 5, 10]. Although many novel explosive compounds have been developed, TNT is still one of the most widely used explosives [10, 34] and according to these facts also the most commonly encountered explosive in soil contamination [4, 9, 10]. It is a predominant contaminant at ammunition plants, testing facilities and military zones [11]. A relatively high number of contaminated sites are located in Germany and in the United States [1]. The U.S. Army has estimated that over 1.2 million tons of soil has been contaminated with explosives [4] and one study showed that TNT occurs in ca. 80% of the contaminated soil samples obtained from U.S. military sites [10]. The concentration of TNT in “hot spots” of contaminated soil can reach 50 g kg^{-1} soil, with the highest levels of TNT contamination directly located at or near the soil surface [4]. Another source mentions that the level of TNT in contaminated soil may vary from trace levels up to 14 g kg^{-1} soil, which is close to its explosive level [11]. TNT can be found as chunks of weathered crystals, tiny crystals embedded in the soil matrix and TNT molecules adsorbed at the soil surface [8].

TNT is not only one of the most stable explosives contaminating soils, it can also reach the water surface by runoff or leaching into ground water,

resulting in contamination of streams and aquifers [3, 4, 6, 11 - 13]. In addition, aquatic environments are contaminated with unexploded ordnance and dumped ammunition [12]. As mentioned above, TNT occurrence in the environment is influenced by its octanol-water partition coefficient [4]. In streams and groundwater it is presented according to its solubility [8] and is absent in the atmosphere due to its low vapour pressure [4].

TNT is considered to be toxic and mutagenic due to a wide range of microorganisms [10], aquatic organisms, terrestrial species, mammals and human monocytes [11]. Several studies show the diversity of toxicity and sensitivity to different tested organisms. Toxic doses can also vary depending to the exposure conditions and times [4]. One of the frequently used indicators in aquatic organisms is bioconcentration, which defines the accumulation of waterborne chemicals by animals through non-dietary routes. For example, the bioconcentration factors (indicator of chemical bioconcentration) determined for fish were reported to be relatively low in TNT. Despite this observation for parent compounds, TNT biotransformation products can be accumulated to significant levels [12]. In addition to the toxicity of TNT, its metabolites are equally or more toxic than the parent compound [6]. On the other hand several microorganisms have been described as being able to use nitroaromatic compounds as nitrogen and/or carbon as their energy source [1] and several of them are chemotactic towards TNT and its accompanying impurities [3].

Due to the risks associated with TNT, remediation of soil and water contaminated by it is more than necessary. Various physical techniques for TNT removal such as incineration, treatment with activated carbon, alkaline hydrolysis and surfactant-enhanced washing have been used [1, 4, 6, 10, 11]. However, most of these methods are very expensive, may cause serious harm to ecosystems and often require additional ex-situ treatment [4, 6, 10, 11]. Bioremediation methods such as composting, slurry-phase treatment and “land farming” are more economic and more environmentally safe alternatives for treatment of TNT-contaminated sites. However, biological methods require longer times of degradation of TNT and more toxic products are often produced during the treatment [4, 10, 11]. Various microorganisms have been reported to be competent in metabolizing TNT such as certain strains of gram-negative

bacteria, gram-positive bacteria, white rot fungi and basidiomycetes under aerobic conditions as well as anaerobic bacteria [11]. Aerobic bacteria tend to transform the TNT molecule by reducing one or two nitro groups, whereas the third group requires anaerobic conditions [4]. Amino-derivatives such as 4-amino-2,6-dinitrotoluene (4-ADNT) and 2-amino-4,6-dinitrotoluene (2-ADNT), which result from partial microbiological degradation are more stable in the environment and more mobile [1, 4]. It has also been reported that they react between themselves, in the presence of oxygen, to form a compound, which causes a higher rate of mutation than TNT itself [4]. Consequently scientists are still trying to find appropriate and effective remediation strategies in explosive-contaminated sites [1, 4, 6, 10, 11].

2.1.5 Effects on animals and humans

According to the toxicity of TNT [1, 3, 4, 6, 8, 11] its wide spread occurrence in soil and water [1, 4, 6, 8 – 13] and low biodegradability [4] it is important to mention its effects on animals and humans because TNT may cause a risk to health. TNT is not only toxic; it is also mutagenic and considered to be a potential human carcinogen [2, 3, 6, 8]. In addition, its metabolites are equally or more toxic than the parent compound [6]. TNT can be absorbed via respiration, ingestion and through intact skin [2, 10]. According to some studies it is known that after it enters the organism TNT is rapidly and extensively metabolized as evidenced by at most only trace amounts of native substance in the urine of humans and animals [2].

Radiolabeled TNT in animal models has been shown to distribute into the blood, lungs, brain, liver, kidneys, spleen and skeletal muscles. Two metabolic routes have been found, one is by the reduction of the TNT nitro groups to aminotoluenes followed by their oxidation to hydroxyarylamines via cytochrome P450, the other is by the oxidation of the TNT methyl group in position 1 to benzyl alcohol or benzoic acid. Benzyl alcohol and hydroxyarylamine conjugated with sulfuryl, glucuronide, and acetyl moieties are at the end of both metabolic routes. The major urine metabolites detected from this process are aminotoluenes and amino-nitrocresol [2].

Some of the common symptoms of TNT acute toxicity are severe headaches, nausea and cyanosis [10]. Longer exposure to TNT can increase the incidence of anemia and liver damage [2, 10, 11]. Several studies have shown that aplastic anemia is caused by affection of the erythropoetic line by TNT and/or its toxic metabolite, which leads to early marrow hyperplasia followed by hypoplasia. Hemolytic anemia is caused by the production of methemoglobin leading to oxidative damage to red blood cells, which mostly yield hemolysis in the spleen. TNT has been associated not only with anemia and hepatic necrosis but also with cataracts, dermatitis, low sperm count [2, 11] and gastrointestinal distress [11]. TNT is therefore classified as group C (possible human carcinogen) by the U.S. Environmental Protection Agency (EPA) [11, 13] with a drinking water equivalent level of 20 mg L^{-1} [11] and a lifetime health advisory level of 2 µg L^{-1} [4, 6, 13].

2.2 Lateral Flow Immunoassay

2.2.1 General informations

The lateral flow assay, also called the immunochromatographic assay or the strip assay [14, 15], mostly means prefabricated strips of a carrier material containing dry reagents that are activated by adding the fluid sample [16, 17], has a user-friendly format thanks to its simplification and rapid on-site testing [15, 17 - 21]. LFIA is designed especially for single use at point of care/need, i.e. outside the laboratory [16, 17] and gives a yes/no response [17, 22]. In this visual qualitative assay the color intensity of the test lines must be high enough to be seen and to distinguish whether there is a difference in color intensity between the negative control and a sample [23]. Results usually come within 10–20 min [16, 18, 19]. The current generation of LFIAs has high sensitivity, selectivity and ease of use [16, 17, 19, 23]. In these days it is a popular diagnostic tool because it eliminates the need for trained personnel and expensive equipment [15, 17, 20].

Development of the LF test strip was a result of a need for some rapid diagnostic method evaluated without laboratory instrumentation. The history of lateral flow technique begins with the discovery of the antibody–antigen immunoassay reaction by combining this reaction with the principles of thin layer and paper chromatography. LFIA is based on latex agglutination assay, which was developed in 1956 by Plotz and Singer. In 1987 Robert Rosenstein for Becton Dickinson & Co., Keith May for Unilever and David Charlton for Carter Wallace filed U.S. patents on the basics of the LF platform. The first lateral flow products were introduced to the market in the late 1980s. In recent years many commercially available readers for quantitative assay had begun to appear [17].

Basic LFIA is a qualitative assay in which the results, meaning the diference between positive and negative samples, could be easily distinguished with the naked eye [15, 18, 19]. But it can be developed to a semi-quantitative and, in limited cases, fully quantitative assay. The traditional LFIAs usually use colored particles such as colloidal gold or latex particles as labels to enable easy observation of results via color change. The use of many new materials as labels, including up-converting phosphor particles, semiconductor

quantum dots, liposome beads, carbon nanotubes and other organic or inorganic nanoparticles [17, 24] confer the objectivity of the result to LFIA, which do not depend on the interpretation of the operator [17, 25]. Materials of labels can all improve the quantitative capability or decrease the limit of detection (LOD) of LFIAs [17, 24]. In quantitative assay results were mostly reported by readers through measuring reflected light or fluorescence [17, 21]. Then quantification of results can be performed by using a standard curve as an external standard but an internal standard can also be used [17]. LFIA can be developed to a one-step multi-target detection method by coated two or more test lines on the LF strip in parallel. Then each line corresponding to one target analyte visible simultaneously [16 – 19, 24]. For better distinguishing of the results latex can be used as a label that is available in multiple colours [17].

2.2.2 Components of the test strip

The lateral-flow strip consists of four elements: a sample pad, a conjugate pad, a membrane and an absorbent pad (**Figure 2**) [17, 21]. The sample migrates from the sample application pad in the conjugate pad and goes through along the membrane where there are the test and control lines [16, 17].

The most important part of the LF strip is a porous membrane due to the fact that it must absorb the conjugate and sample from the conjugate pad, carry them consistently to the reaction area, allow the reaction at the test and control lines and allow excess fluids, reactants and label not bound to exit when the test strip is run. In order to function as a reaction matrix it must be hydrophilic, thus it is made hydrophilic by the addition of rewetting agents (surfactants) during the membrane production process [17]. It is often thin and fragile so it is attached to a plastic or nylon basic layer (backing) to allow cutting and handling. In addition, robustness is achieved by housing the strips in a plastic holder [16, 17, 22] where only the sample application window and the reading window are exposed [16, 22] but it is also posssible to use it as stand-alone test strips [17, 22]. The current membrane strips are produced mainly from nitrocelulose [14, 16, 17, 27], then a little less from nylon [16, 17],

polyethersulfone, polyethylene or fused silica [16]. On the membrane specific biological component of the assay has been immobilized (antibody or antigen) [17, 20, 26 - 28].

At the proximal end of the strip a sample application pad is provided [17]. It helps and controlls distribution of the sample onto the conjugate pad [16, 17, 22]. The sample application pad is usually made of cellulose or cross-linked silica [16, 17].

In close contact with the strip material and the sample application pad is the conjugate pad, made of cross-linked silica [16, 17, 22] or polyester [17, 22]. It's the most important role is to uniformly deliver the sample and the label to the membrane [22]. On this pad are usually dried labelled recognition elements. After addition of the liquid sample the label solubilizes and is released from the conjugate pad, it can begin specific interactions between antigens and antibodies [16, 17, 22]. The conjugate pad contains of buffer chemicals, blocking reagents and stabilizers [27]. It is also possible to carry out the test without a sample pad and to insert the conjugate pad directly in the sample solution, like a dipstick test [19].

Labels are very important particles due to the fact that without them it is not possible to measure the amount of analyte in the sample. Only the capture analyte on the test line bound to the labelled recognition element produces the signal [22]. Labels can either be separately mixed with the sample or are directly bound to the conjugate pad [27] and after running the test they allow visualisation and according to the label used even quantitation of the response [16, 17]. They are made of coloured or fluorescent nanoparticles with sizes of 15–800 nm. This size allows an unobstructed flow through the membrane [16]. The material they are often made of is colloidal gold or latex [16, 17, 22, 27], less frequently selenium [16], carbon or liposomes are used [16, 17, 22, 27]. Colloidal gold, carbon and selenium are specifically designed for visual detection [16, 17]. In latex nanoparticles can be encapsulated color dyes including fluorescent dyes and paramagnetic media. Among the fluorescent labels belong the xanthene dyes (such as fluorescein and Texas Red), the long-wavelength Cyanine (Cy) dyes, lanthanide complexes and quantum dots. The upconverting phosphor technology is based

on luminescence [17]. In liposomes coloured dyes, fluorescent dyes, enzymes or electroactive compounds can be incorporated [16, 17].

At least two lines are sprayed on the strip: a test line and a control line. At the test line the recognition of the sample analyte and the labelled antibody by an immobilized ligand (e.g. antibodies) will result in an appropriate response [14 - 29]. A response at the control line confirms a proper flow of the liquid through the strip [16, 20, 25, 27]. If the control line does not appear the test is considered to be invalid [17 - 19, 29]. More test lines can be applied allowing multianalyte testing or semiquantitative evaluation of the response [16, 24].

The liquid moves up because of the capillary force of the strip material but to maintain a flow an absorbent pad is attached at the distal end of the strip. This absorbent pad keeps the flow and will wick the liquid to the end of the strip so it can increase total volume of sample that enters the LF strip [16, 17, 22, 27]. Most frequently it is made from celulose [16, 17, 22].

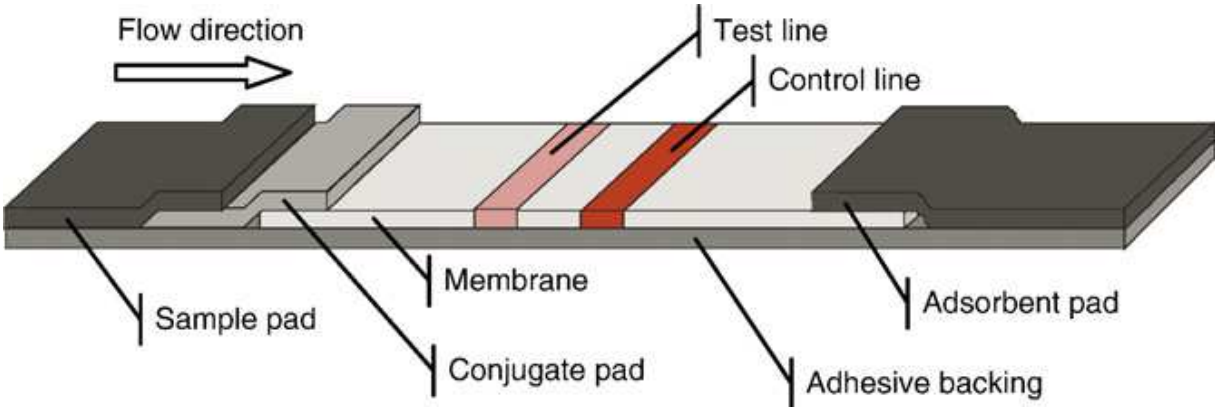

Figure 2 Schematic view of a typical LFIA

A porous membrane is mounted on an adhesive backing. A specific ligand (e.g., antibodies) is immobilized on the test line. On the control line, capture ligands for the labelled antibodies are bound. Sample and conjugate pad enable the equal distribution of the liquid sample and serve as a reservoir for the assay reagents. The adsorbent pad takes up excess liquid and ensures sufficient flow-through. [27]

2.2.3 Principles and formats of LFIA

Essential in the current LFIA formats is the movement of a liquid sample or its extract containing the analyte of interest along a strip by capillary action. During this process sample passes various zones where specific molecules have been attached that perform more or less specific interactions with the analyte [16, 17, 20, 26, 27]. Antibodies against the analyte are used for recognition [16, 17, 27, 28]. Usually they are immobilized at a predetermined location on the membrane called test line [17, 20, 22, 26 - 28], they can be also used as conjugate on the label or both, depending on the assay design [22]. Earlier use of polyclonal antibodies provides easily obtainable results but have the limitations of high cross-reactivity, meaning that the antibody does not have enough specificity and can also bind another antigen than at first intended. Due to this fact a fals positive result will be obtained. The use of monoclonal antibodies has substantially increased the specificity and sensitivity of the immunoassay [17]. Several formats have been described for LFIAs. These formats are chosen depending on the analyte [16, 17].

2.2.3.1 Sandwich assay

For larger analytes with more than one epitope, such as LH, hCG, and HIV, the sandwich (non-competitive, direct) format is applicable [14, 16, 17, 28]. In this format the test line is prepared using one antianalyte-specific antibody. The conjugate release pad contains a second, another, labelled, antianalyte antibody [14, 16]. Analyte present in the sample will be bound during the initial chromatographic process to the secondary antibody. The free epitope of analyte can bind to the immobilised antibody at the test line [14, 16, 20, 26, 28]. A positive result is indicated by the presence of a test line because secondary antibody bound with the analyte is dyed by label. In almost all cases the label in excess is compared to the sample so some of the conjugated particles will not be captured at the test line and will continue through the strip to the control line [17]. The response is directly proportional to the amount of analyte in the sample; a scheme is depicted in **Figure 3** [16, 28].

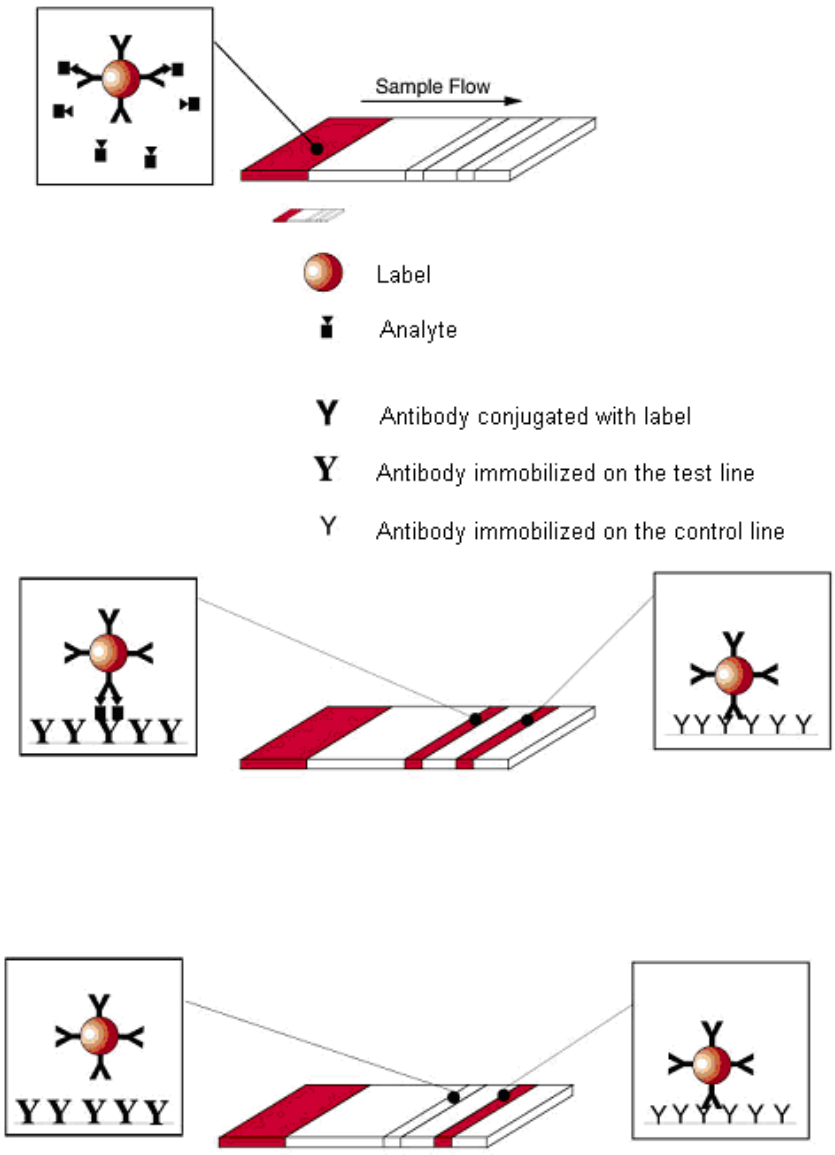

Figure 3 Scheme of sandwich LFIA

The response is directly proportional to the amount of analyte in the sample, at the top is positive test, at the bottom is negative test [16].

2.2.3.2 Competetive assay

When the analyte is of low molecular mass or moreover has only one epitope, i.e. a hapten, it cannot simultaneously bind to two antibodies [17] so only the competitive design is permitted [16, 17, 28]. Two formats of this design are possible:

(1) In the first format molecules are usually smaller than 6,000 kD [17]. The antibody is sprayed onto the test line, a mixture of sample analyte *A* and labelled analyte (reporter) *P* is applied at the conjugate pad. The sample analyte and labelled analyte compete for binding sites on the antibody at the test line [16, 17, 20, 28] to form complexes R_TA or R_TP with test ligands R_T. Once the ligand has formed the complex R_TA it is no longer able to bind with a reporter. Only the reporters (but not the target analyte) can bind to the control line R_C to form the complex R_CP. Thus the target analytes compete with the reporters for binding sites. This format can be refered as RPA to indicate that both reporter particles *P* and target analyte *A* can bind to the test ligand *R* [20].

(2) In the second format antigens of smaller sizes (e.g., a hapten), particularly those smaller than 1 kD, do not make optimal immunogens. They diffuse from the site of aplication very quickly and are cleared from the system by mechanisms other than the immunoreaction. To prevent this adverse effect these antigens are coupled to carrier proteins, often keyhole limpet hemocyanin (KLH) or ovalbumin (OVA) [17]. An analyte-protein conjugate R_T is sprayed at the test line and a mixture of labelled antibody (reporter) *P* and sample analyte *A* is applied at the conjugate pad [16, 20]. The reporter particles can bind with either the target analyte or the test ligand R_T. Once the target analyte *A* binds to the reporter *P* to form the complex *PA* the reporter cannot bind to the ligand R_T. It can, however, still bind to the control line to form the complex R_CPA. The free reporters *P*, but not the complex *PA*, can bind to the immobilized test ligand R_T at the capture zone to form the complex $R_T P$. The free reporters *P* can also bind to the immobilized ligand R_C at the control line to form the complex R_CP. The target analytes can bind neither to the test ligand nor to the control ligand. This format can be referend to as RPNA to indicate that the reporter particles *P* but not the target analyte *A* can bind to the test ligand *R* [20].

The preferred layout is dependant on the particular application. In the competitive LFIA format the response is negatively correlated to the analyte concentration (i.e. more analyte presents less signal, thus absence of the test line; no analyte gives the highest signal, thus clearly visible test line) [16, 17]. A control line should still form [16 - 20, 22]. When a high

amount of analyte is present in the sample it will be able to block more effectively the capture of labeled analyte onto the test line, so a major part of the label will be captured in control line. When less amount of the analyte is present, labelled analyte will be captured in the test line and a minor part of this conjugate with label will continue to flow toward the control line, so it can be less visible than test line [25, 28]. A typical scheme of the competitive design and the response using option 2 is depicted in **Figure 4** [16].

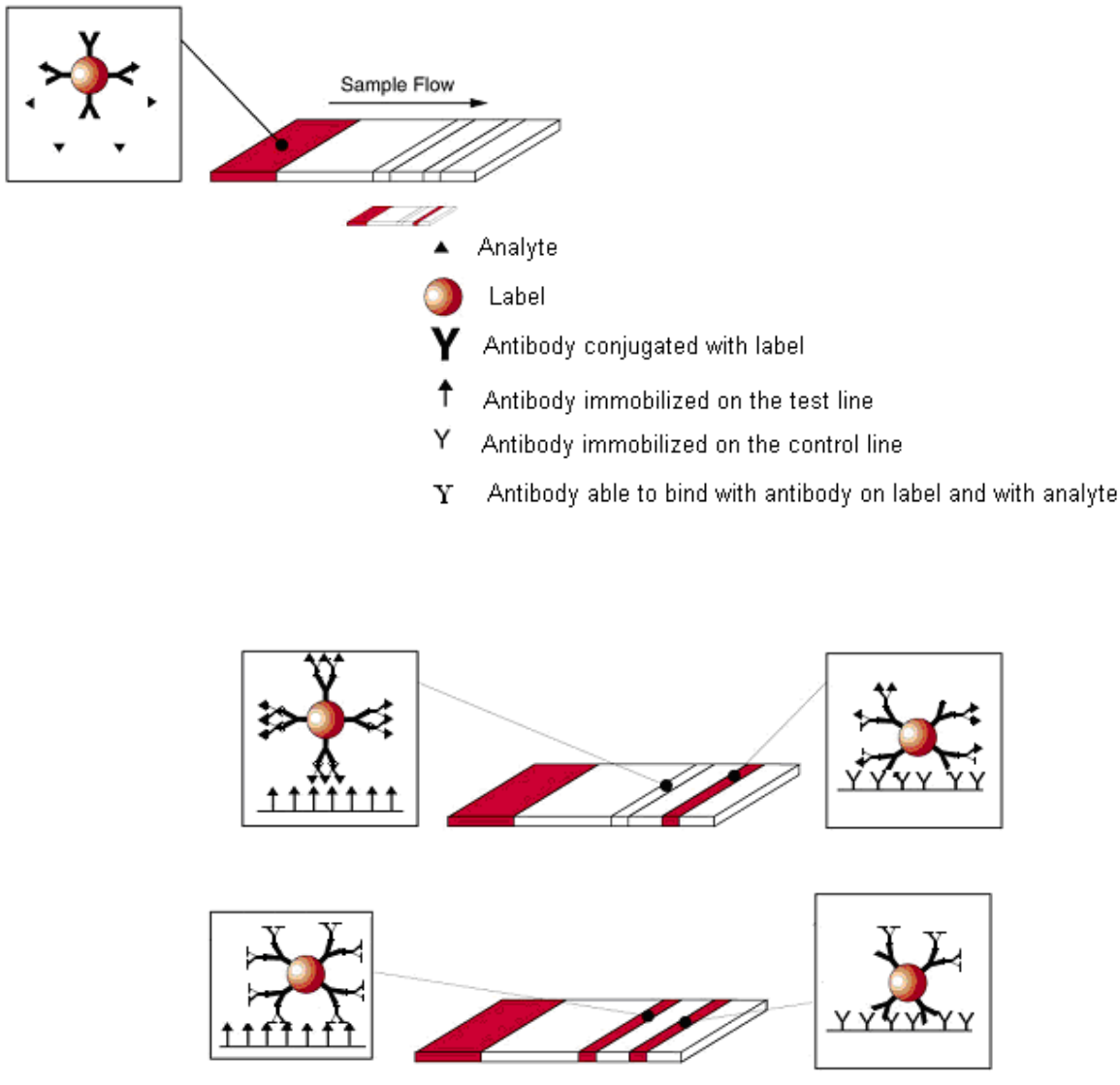

Figure 4 Scheme of competitive LFIA

The response is negatively correlated to the amount of analyte in the sample, at the top is positive test, at the bottom is negative test [16].

2.2.4 Critical parameters for sensitivity of the assay

2.2.4.1 Membrane's material

The porous membrane constitutes the biggest and probably the most important part of the test system. The pore size and the material provide the transport of the liquid [16, 22]. Various pore sizes are available on the current market. Larger pore sizes will widen the test line and the sensitivity of the test will decrease because faint lines can be missed [16]. Typically a test line is 1 mm wide [22]. The pores are not randomly distributed because of the manufacturing process. Due to this fact a better description of the strip material should be „capillary flow time" or „wicking rate'', i.e., the time required for the fluid to travel onto the strip material with defined width [16, 17, 22] and is expressed in seconds per centimetre [16, 22]. Further, an important parameter is speed with which the complex of the sample analyte and the label are transported through the membrane allowing optimal reaction time [16]. With lower speed it is possible reach higher sensitivity but increasing run time can produce false positive results. As was mentioned to make membrane hydrophilic surfactants are used but not every protein is compatible with every surfactant [17]. The protein-binding capacity of a membrane is the parameter that is determined by the amount of polymer surface area that is settled by pore size, porosity, thickness and structural characteristic and also affect the sensitivity of the diagnostic test. Immunologically active proteins should be consistently immobilized to test and control lines. Nitrocellulose membrane binds proteins through a combination of electrostatic, hydrogen and hydrophobic forces [17, 22]. Majority of the proteins lose much of their immunological activity after binding passively to the membrane surface due to their inability to bind covalently or directionally to nitrocelulose [16, 17]. Humidity should be optimized for binding (25–50% relative humidity at room temperature) [17].

2.2.4.2 Material of the sample pad

The sample pad must be able to accept all of the sample volume applied to it in a controlled way, preventiv flooding of the device [17, 22]. The pores

in the sample pad can be symmetrically, homogeneously or inhomogeneously distributed or they can be asymmetrically distributed, providing an initial filter to remove particulates or red blood cells [16]. The sample pad can also change the pH of the sample or actively bind sample components that can interfere with the assay [17]. To increase the sample viscosity, to increase the reaction time at the conjugate pad or even to chemically modify the sample for binding at the test line it is possible to impregnate the sample pad with, e.g. pH buffer, proteins (such as albumin), surfactants (e.g. Tween 20 at a very low concentration), blocking reagents (if required), additives and other reagents [16, 17, 22].

2.2.4.3 Material of the conjugate pad

The conjugate pad should provide a uniform transfer of the sample and of the label to the membrane [22]. Sensitivity can be negatively affected according to poor conjugate mixing and release from the conjugate pad. Whether it is better to have a fast or slow release depends on the test system but release must be consistent. As well as strip material the conjugate pad must also be hydrophilic. Due to this fact it is immersed in a solution of proteins, polymers and surfactants [17].

2.2.4.4 Material and size of the label

For visualization of the response labels are used. The most common material of the labels is colloidal gold or latex [16, 17, 22, 27]. An important requirement for the nanoparticle labels is their colloidal stability in solution [16, 22]. Stability is achieved when the particles are monodisperse with a consistent spherical shape [22]. A stable suspension ensures a uniform distribution of the conjugate in the conjugate pad [16] and follows through the membrane. Smaller particles would move faster than larger particles [22]. In visually analyzed assays a better sensitivity is possible to be obtained using colloidal gold rather than colored latex particles. Because of its smaller size higher density of gold particles can be achieved on the test line, in addition it has higher color intensity than colored latex particles, which allows better

distinguishing of low positive results [17]. Size of colloidal gold particles is typically 40 nm in diameter [16, 17]. Higher sensitivity was observed using larger gold particles but the stability of the colloid decreases with particles over 40 nm [16]. On the other hand colored latex particles can be produced in multiple colors and also darker colors so higher sensitivity can be reached by greater contrast against the white background of the test strip. Latex particles are about 100–300 µm in size. By using fluorescent particles or paramagnetic particles it is often possible to generate higher sensitivity in reader-based assays [17].

2.2.4.5 Recognition element

The sensitivity of the LFIA depends on the affinity of the specific antibodies [16, 17, 22]. Their activity can be affected via the conjugation methods used during the labeling procedures [17]. Specific antibodies (primary antibodies) can be labelled but it is often necessary to use high concentration of this expensive material. Secondary antispecies antibodies (often less expensive) can also be labelled and the primary antibodies can be titrated for optimal response. The optimal ratio between concentration of the label and the recognition element is aimed at optimal sensitivity of the assay [16]. Concentration of the analyte in the sample could also be a problem. When the presence of the analyte is too high it can appear as a „high dose hook effect“. There must be an excess of antibodies in the system, both capture on the lines and label in the conjugate pad, relative to the analyte being detected. Normally the dose–response curve has a positive slope but if there is a redundancy the analyte dose–response curve will plateau and further increase of the analyte will cause the slope to become negative [17]. The analyte concentration over the critical value leads to reduction of the signal on the strip test [20]. The prevention of hook effects can be avoided by sample pretreatment reducing its concentration. Less concentration of the analyte is also related to the limit of detection. Specificity is influenced by the cross-reactivity of the antibodies, the polyclonal antibodies have in general lower specificity than monoclonal antibodies. Cross-reactivity could lead to false positive results [17]. Threat for the antigen-antibody interaction could also be

steric hindrance [16]. This situation can occur when antigens are relatively small and should bind to multiple antibodies because there is not enough physical space [17].

2.2.4.6 Position of the test line

The position of the test line has significant impact on the performance and sensitivity of the assay. More interaction time is available when the test line is placed farther from the origin due to the lower flow rate and thus higher effective concentration of anlayte in the sample passes through the capture zone. An optimum location is usually obtained as the capillary flow rate decreases exponentially with the distance [16, 22].

2.2.4.7 Material of the absorbent pad

The absorbent pad helps increase the total volume of the sample that can enter the test strip [22] and should not release fluid back into the assay because false positive results can occur [17]. The absorbent pad is used to control the volume of the sample that passes through the capture line. If the absorbent pad would not be used, the amount of the sample and labelled analyte, and also signal, will decrease on the test line [22].

2.2.4.8 Sensitivity

Sensitivity is the ability of a method to detect truly positive samples as positive [19]. There are two main definitions of sensitivity:

(1) ‘‘Response per unit ligand’’ is the slope of the dose–response curve and is primarily applicable in quantitative assays.

(2) ‘‘The lowest level of non-zero ligand reliability’’ is measured as the lowest detectable dose (LDD), also called limit of detection (LOD), which is more applicable in qualitative assays and is the most commonly applied definition in LFIAs where there is need to distinguish between positive and negative result.

Which definition of sensitivity is better to be used depends on the characteristics of the assay [17].

2.2.5 LFIA, NALFIA, NALF

It is possible to divide the lateral flow in three set-ups according to the interacting analytes. When only antibodies are used as recognition elements the tests are called “lateral flow immunoassays” (LFIA). Another set-up of when the analyte is an amplified double-stranded nucleic acid sequence (ds-amplicon) and recognition element of the analyte is a tag-specific antibody is also possible and is called “nucleic acid lateral flow immunoassay” (NALFIA). In this layout the nucleic acid used to be amplified by PCR. Specific nucleic acid hybridisation of amplicons with immobilised complementary probes is another option and is called “nucleic acid lateral flow assay” (NALF) [16].

2.2.6 Usage

Thanks to their simplicity, rapidity, cost-effectiveness and user-friendly format [16 – 19, 29] lateral flow tests have achieved an increasingly wider utilization in lots of fields. The pregnancy test (human chorionic gonadotropin) is the well-known for being available to broad public these days in all pharmacies and supermarkets in developed countries. Other LF sandwich assays can also serve for the detection of infectious diseases (HIV, hepatitis B and C, H. pylori, *Streptococcus A* and *B*), cardiac markers, (tropoinin C, creatinine kinase - MB, myoglobin) and malignancies (prostate-specific antigen). Hormones, therapeutic drugs and abusive drugs can be determined using competitive assay [17, 21, 22, 28, 29]. LFIAs can be used to test just about any biological sample, including urine, tears, sweat, saliva, serum, plasma, whole blood and biopsied tissue and fluids [16, 17, 28]. As mentioned above it is possible to use it for testing everywhere: in the consulting room of the general practitioner, in the emergency ward of hospital, screening for abusive drugs for law enforcement [16, 17], in the veterinary medicine to screen commercial livestock and household pets for a number of medical conditions [17], absence of contaminants in food/feed, seed, seedlings [16, 17, 22] and water [17],

forensics analysis and tests related to biowarfare, where fast results are important [16, 17]. LFIA also spreads in newer areas such as molecular diagnostics and theranostics [17].

2.2.7 Advantages and disadvantages

Advantages of LFIA are well known. It is popular thanks to its user-friendly format with a rapid yes/no response that it provides [16 – 20, 29]. Using new materials as labels it can also give semiquantitative and in some cases even quantitative results [16, 17, 24]. This relatively inexpensive test needs only low sample volume for a single analysis and can be tested at a point of care/need by untrained personnel [16, 17, 19, 29]. During the development process was obtained good sensitivity, selectivity and specificity [16, 17, 19]. It is ideally suited for on-site testing and has a long term stability over a wide range of climates [16, 17, 29] (the lifetime can be of 12–24 months often without refrigeration) [17]. On the other hand LFIA also has disadvantages. In basic format LFIA is a qualitative assay with a simple positive/negative response but in these days higher requirements are put on it. Mostly sensitivity and test-to-test reproducibility challenge the limits of its applications in quantitative systems [16, 17]. In this assay format no washing step is possible. The sample pretreatment is obligatory when the sample is not fluid. Untrained personnel can make a mistake in distinguishing between a positive and a negative response and in addition using competitive format of test, response negatively correlates to the concentration [16]. LFIA can suffer from high dose hook effect, so above the critical concentration the anlayte can not recognize the real amount of analyte [17, 20].

2.2.8 Comparison with other immunoassays

The LFIA technology is based on an antigen-antibody reaction as other immunoassays, such as enzyme-linked immunosorbent assay (ELISA) or western blots [16, 22, 27]. At the beginning of the development of LFIA it connected chromatography methods with the same components as in enzyme immunoassays, antibodies as recognition elements and enzymes as labels.

Nowadays enzymes in LFIA were replaced by particulate labels [16]. In recent years with the introduction of a new LF method its sensitivity and specificity is mostly referenced to ELISA [16, 30]. ELISA is a quantitative biochemical technique used mainly in immunology to detect the presence of an antigen or an antibody in a sample [31, 32]. Antibodies or antigens used for detection are attached to a solid phase, usually a polystyrene microtiter plate with an 8x12-well format [32, 33]. At the end of the test the plate is made by adding an enzymatic substrate to produce a signal that is then measured and indicates indirectly the amount of antigen in the sample [31]. According to the enzyme used for visualization, ELISA is divided in fluorescent and chemiluminescent. Comparing the ELISA lateral flow does not require laboratory equipment and results are obtained more quickly. On the other hand LFIA suffer from lower sensitivity [16]. According to this fact LFIA is useful when a rapid result is necessary that gives the answer that the sample contains more or less amount of the analyte than the limited concentration of this analyte. When a quantitative result is needed the ELISA is more successful.

3. Practical part

3.1 Materials and methods

3.1.1 Reagents

3.1.1.1 Chemicals

Tetrachloroauric acid ($HAuCl_4$)

Trisodium citrate

Sodium azide

Sodium chloride

Standard TNT (0.1 mg mL^{-1}) in MetOH:Acetonitrile (50:50, v/v) (AccuStandard, New Haven, USA)

3.1.1.2 Buffers

Borate buffer 0.2 M pH 8.5

$Na_2B_4O_7$*10$H_2$0.....19.07 g L-1 0.05 M

H_3BO_3.....12.37 g L^{-1} 0.2M

H_2O bidistilled.....1000 mL

Borate buffer 20 mM

$Na_2B_4O_7$*10$H_2$0.....1.907 g L^{-1} 0.05 M

H_3BO_3.....1.237 g L^{-1} 0.2M

H_2O bidistilled.....1000 mL

Borate buffer 20 mM + 1% BSA

$Na_2B_4O_7$*10$H_2$0.....1.907 g L^{-1} 0.05 M

H_3BO_3.....1.237 g L^{-1} 0.2M

BSA.....10 g L^{-1}

H_2O bidistilled.....1000 mL

Borate buffer 2 mM + 1% BSA

$Na_2B_4O_7$*10$H_2$0.....0.1907 g L^{-1} 0.05 M

H_3BO_3.....0.1237 g L^{-1} 0.2M

BSA.....10 g L^{-1}

H_2O bidistilled.....1000 mL

Borate buffer 20 mM + 1% BSA + 2% sucrose + 0.25% Tween 20 + 0.05% sodium azide

$Na_2B_4O_7$*10$H_2$0.....1.907 g L^{-1} 0.05 M

H_3BO_3.....1.237 g L^{-1} 0.2M

BSA.....10 g L^{-1}

Sucrose.....20 g L^{-1}

Tween 20.....2.5 g L^{-1}

Sodium azide.....0.5 g L^{-1}

H_2O bidistilled.....1000 mL

Coating (carbonate) buffer 0.05 M pH 9.6

Na_2CO_3.....1.59 g L^{-1} 15 mM

$NaHCO_3$.....2.93 g L^{-1} 35 mM

H_2O bidistilled.....1000 mL

Phosfate buffered saline (PBS) 1X 0.1 M pH 7.4

NaCl.....7.950 g L^{-1} 137 mM

KCl.....0.201 g L^{-1} 2.7 mM

KH_2PO_4.....0.204 g L^{-1} 1.5 mM

Na_2HPO_4*2H_2O.....1.423 g L^{-1} 8.0 mM

H_2O bidistilled.....1000 mL

3.1.1.3 Reagents for immunoassay

Primary antibody

As a primary antibody was used Mouse anti-TNT monoclonal antibody A1.1.1 purchased from Strategic Diagnostic (Newark DE, USA).

Secondary antibody

As a secondary antibody was used rabbit anti-mouse (RAM) purchased from Sigma-Aldrich (St. Louis, MO, USA). RAM was used for conjugation with colloidal gold.

Tertiary antibody

As a tertiary antibody was used goat anti-rabbit (GAR) purchased from Sigma-Aldrich (St. Louis, MO, USA). GAR was used for covering control line.

Conjugate on the test line

Trinitrobenzenesulfonate conjugated with ovalbumine (TNBS-OVA) was used for covering test line. The hapten (TNBS) was conjugated to ovalbumine in previous studies.

3.1.2 Instruments

Shaker (Lumac mts2, IKA® Werke GmbH & Co. KG, Germany) was used for uniform conjugation between colloidal gold and secondary antibody.

Sorvall RC2-B Superspeed centrifuge (Thermo Fisher Scientific, USA) was used to prepare conjugate.

Figure 5 Sorvall RC2-B Superspeed centrifuge

Easy Printer LPM02 printer device (Advanced Sensor Systems, India) was used to coat nitrocelulose membrane with test and control lines.

Nitrocellulose membrane HIFlow Plus HFB1200225 were obtained from Millipore (Billerica, MA, USA).

Figure 6 Easy Printer LPM02 printer

3.1.3 Methods

3.1.3.1 Real sample and their extraction

The sample was collected from metal shelf surface after an experimental explosion. For this test explosion, controlled by police, was used 20 g of TNT. This amount was inserted inside a video cassette and after that placed into an A4 envelope to simulate a mail bomb. This envelope was fixed on a metal shelf and simulated bomb was activated by an N.8 electric blasting cap [34]. The metal shelf was used during this project for collecting samples. It is an assumption that some amount of TNT is caught on the shelf surface after the explosion.

For collecting samples were chosen four different areas on the shelf surface. From each area, labeled A, B, C and D, were collected two different samples. Each sample was wiped off 1 cm^2 area using cotton swabs wetted with methanol. Each swab was then dipped in 500 µL of methanol and for extraction was used intensive shaking in hand for 3 minutes.

Figure 7 Collecting samples from areas A, B, C and D with cotton swab wetted with methanol

3.1.3.2 Colloidal gold preparation

Gold nanoparticles were obtained according to the method of Frens (1973) [34]. Previously prepared 50 mL of 0.01% tetrachloroauric acid was heated to boiling. 500 µL of 1% trisodium citrate was prepared and when

tetrachloroauric acid started to boil, trisodium citrate solution was added under constant stirring. Final solution was left to boil until it changed colour from yellow to blue and finally to cherry red. This process (change in colour) indicated formation of monodisperse spherical particles, 45 nm in size.

Colloidal gold was left to cool. The pH was adjusted to value 7.5 with coating buffer (0.05 M, pH= 9.6) and 0.05% sodium azide solution was prepared and added inside the colloidal gold. The colloidal gold was stored at temperature 4℃.

Figure 8 Boiling colloidal gold with addition of trisodium citrate changed colour to cherry red.

3.1.3.3 Secondary antibody titration

Optimal ratio between colloidal gold and secondary antibody is necessary to find. This optimal ratio is possible to find by titration of antibody. In **Table 2** is mentioned amount of each solution added inside the test tube.

Colloidal gold was mixed with RAM 0.1 mg mL^{-1} and borate buffer 20 mM. After 10 minutes 10% sodium chloride solution was added. The test tube No. 1 is without secondary antibody and it was considered as control tube. The reagents inside this tube had to change colour after adding sodium chloride solution. Other tubes retained their color that means there was enough

of antibody for conjugation with colloidal gold. Selected ratio in our experiment was 20 µL RAM 0.1 mg mL^{-1} for 1 mL of colloidal gold.

Number of test tube	1	2	3	4
Colloidal gold [µL]	1000	1000	1000	1000
RAM 0.1 mg mL^{-1} [µL]	0	20	40	60
Borate buffer 20 mM pH8.5 [µL]	150	130	110	90
Solution NaCl 10% [µL]	100	100	100	100

Table 2 Amount of solutions inserted in the test tubes

3.1.3.4 Colloidal gold-antibody conjugation

It is possible to conjugate colloidal gold either with primary antibody or with secondary antibody. Rabbit anti-mouse secondary antibody was used for conjugation due to often high price of primary antibody. According to the secondary antibody titration 500 µL of borate buffer (20 mM pH 8.5) and 20 µL of RaM 1 mg mL^{-1} in PBS 1X were added to 5 mL of colloidal gold. This solution had been constantly stirred for 1 hour at room temperature by use of shaker to create a conjugate (rabbit anti mouse colloidal gold).

20 mM borate buffer pH 8.5 containing 1% of BSA was added to rabbit anti mouse colloidal gold (RAM-CG) to block unoccupied places on the secondary antibodies. This solution was centrifuged at 25,000 g for 30 minutes at 10℃. Pure supernatant was removed and the pellet after centrifugation was resuspended in 20 mM borate buffer pH 8.5 containing 1% of BSA and after that centrifuged again at 25,000 g for 30 minutes at 10℃. After this centrifugation the pellet was resuspended in 20 mM borate buffer pH 8.5 containing 1% of BSA, 2% of sucrose, 0.25% of Tween 20, and 0.05% of sodium azide. This solution of final conjugate was stored at 4℃. All centrifugations were carried out in a Sorvall RC2-B Superspeed centrifuge (**Figure 5**).

3.1.3.5 Preparation of the strips

In this step nitrocelulose membrane was coated by test and control line. Solution for creation of the test line was prepared from TNBS-OVA 250 µg mL^{-1} dissolved in PBS 1X. Solution for creation of the control line was prepared by dissolving goat anti-rabbit tertiary antibody 200 µg mL^{-1} also in PBS 1X. 10 cm long piece of nitrocelulose membrane 2.5 cm wide was cut off and then fixed on the Easy Printer LPM02 printer device. 100 µL of each solution were used for coating the strip. The printer device distributes 1 µL of solution per 1 mm line. Test and control lines were created simultaneously by running printer device ten times on the same position. The distance between test and control lines was approximately 0.5 cm. Coating of nitrocellulose membrane is depicted on **Figure 6**. Coated membrane strip was stored overnight at 37°C and then was cut into 20 strips 0.5 cm wide and 2.5 cm long.

3.1.3.6 LFIA procedure

The nitrocelulose membrane strip was fixed with the upper part of the test strip vertically to the pad. The upper part of the test strip was in contact with filter paper which performed function of an absorbent pad. As a conjugate pad was used solution containing: 50 µL of running buffer (PBS 1X containing 1% of BSA), 10 µL of rabbit anti mouse-colloidal gold, and 3 µL of mouse anti-TNT antibody in PBS 1X (1:100, v/v). LFIA was performed by dipping the strip in the well containing the reagents described above. The test proceeded until all the solution had gone from the well throughout the membrane into the filter paper. RAM-CG reacted with mouse anti TNT primary antibody in the solution of simulated conjugate pad and then this complex reacted with hapten conjugate TNBS-OVA on the test line. On the control line RAM-CG bounded on GAR tertiary antibody. After 10 minutes test and control lines were visible, what was the proof of correct function of the LFIA system. This step without sample or standard solution is considered as blank test and gives negative result (two visible lines). Further control of function of the LFIA system was accomplished by adding 1 µL of standard solution of TNT 0.1 mg mL^{-1} in MeOH:Acetonitrile (50:50, v/v). Primary antibody reacted with standard solution of TNT, which is small hapten,

that is why this complex was not able to bind on TNBS-OVA on the test line. RAM-CG reacted with tertiary antibody on the control line like in the previous step. In addition the complex mouse anti TNT-RAM-CG-TNT can also bind with GAR on the control line. The intensity of the test line should be lower than the control line or the test line can even totally disappear. If no control line was present, the test was considered to be invalid.

3.1.3.7 Testing real samples

Extracts of four different samples from area A2, B1, C1 and D1 were tested in several different dilutions. Positions of these areas are depicted on **Figure 9**.

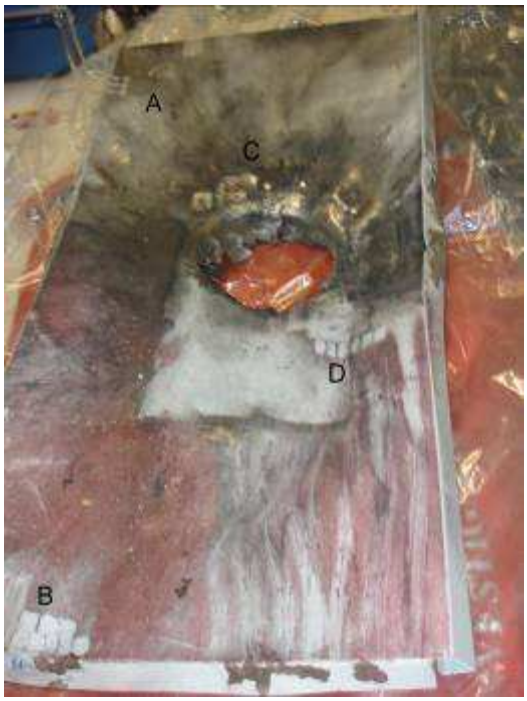

Figure 9 Sample colection areas A, B, C and D

Each sample diluted 1:10 in methanol was added in well containing 50 µL of running buffer (PBS 1X containing 1% of BSA), 10 µL of rabbit anti mouse-colloidal gold, and 3 µL of mouse anti-TNT antibody in PBS 1X (1:100, v/v). According to weak signal (high intensity of the test line), except sample C1, the same amount of each sample in their basic concentrations was used. Sample C1 was tested also in dilution 1:100 in methanol. Increasing

concentration of TNT in sample decreases intensity of the colour of the test line until its complete disappearance. If no control line was present, the test was considered to be invalid.

3.1.3.8 The limit of detection determination

One to distinguish between positive and negative result in LFIA needs to know one important parameter, limit of detection (LOD). This parameter is measured as the lowest detectable dose and is referred to sensitivity of the assay. LOD is determined as the smallest amount of the analyte that cause visible difference between the colour development of the test line on the blank test and the colour development of the test line on the test strip. In this research, previous studies discovered LOD by adding 1 μL of standard TNT at concentrations in the range 100–0.01 μg mL^{-1}. LOD was determined to value 1 μg mL^{-1}.

3.1.3.9 The cross-reactivity determination

The rate of specificity can express parameter called cross-reactivity, which determines if the test gives positive result only in case of presence of required analyte or not. In previous studies of this project several TNT-related compounds were tested and the cross-reactivity was observed for 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. Cross-reactivity is indirectly proportional to the specificity of the system.

3.1.3.10 Comparison with chemiluminescent ELISA

Standard solution of TNT (0.1 mg mL^{-1}) and all of the sample extracts in MeOH from areas A, B, C and D were tested also by chemiluminescent ELISA. Same type of antibodies and also the same format of assay as in the LFIA were used. Results were obtained by luminometer connected with computer. Identified concentrations of analyte in the samples corresponded with results acquired by LFIA.

3.2 Results and discussion

The aim of this research was to develop a rapid on-site method for testing real samples containing TNT with qualitative yes/no response. The previously performed optimization gave information about the best conditions, especially the most suitable antibodies, hapten conjugate immobilized on the test line and the method for extraction of the samples. It is important to validate the correct function of the system for development of an exact immunochromatographic method.

3.2.1 Validation of correct function of the LFIA system

3.2.1.1 The blank test

To validate the correct function of the LFIA system and to obtain the blank test the following solution was used: 50 µL of running buffer (PBS 1X containing 1% of BSA), 10 µL of rabbit anti mouse-colloidal gold, and 3 µL of mouse anti-TNT antibody in PBS 1X (1:100, v/v). In this research the competitive format of assay was used when the target analyte (TNT) was a small hapten. If there is zero concentration of TNT in the reaction medium, mouse anti TNT binds on RAM-CG and this complex binds onto hapten conjugate (TNBS-OVA) immobilized on the test line. Colloidal gold is the label, so it is a carrier of the colour signal. If TNT is not present, the test line has to be clearly visible. GAR immunoglobulin is immobilized on the control line, which is an anti species antibody to the RAM secondary antibody. Since there is an excess of RAM in the reaction medium, one part of the RAM can bind to the GAR (tertiary antibody) present in the control line independently of the presence or absence of the researched analyte in the sample. If the control line is not present the test is considered to be invalid. **Figure 10** shows a negative test described above from this experiment.

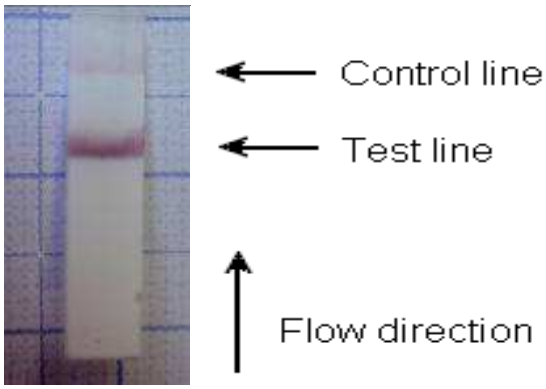

Figure 10 The blank test (50 µL of running buffer, 10 µL of rabbit anti mouse-colloidal gold and 3 µL of mouse anti-TNT antibody in PBS 1X)

3.2.1.2 Standard positive test

The second step in validating the correct function of the LFIA system was performed using a solution containing the same reagents in the same amounts as in the blank test plus 1 µL of standard solution of TNT 0.1 mg mL^{-1} in MeOH:Acetonitrile (50:50, v/v). In this case hapten (TNT) competes with hapten conjugate (TNBS-OVA) for antibody binding sites on primary antibody (mouse anti TNT). Complex mouse anti TNT-RAM-CG-TNT is not able to bind onto TNBS-OVA, so the color of the test line is less intense with respect to the test line of the blank. The decrease of the intensity, until the complete disappearance of the line, depends on the ratio between TNT and mouse anti TNT antibody. All of the RAM-CG is available to bind onto the control line. In a positive test the control line is more intense than in a negative/blank test, since the RAM-anti-TNT antibody complex, which in the negative test will bind the test line, in positive test is free to reach and to bind the control line. Also in this case creation of the control line is a visible confirmation of particle flow of the LFIA test and if the control line is not present the test is considered to be invalid. **Figure 11** shows a positive result from this experiment.

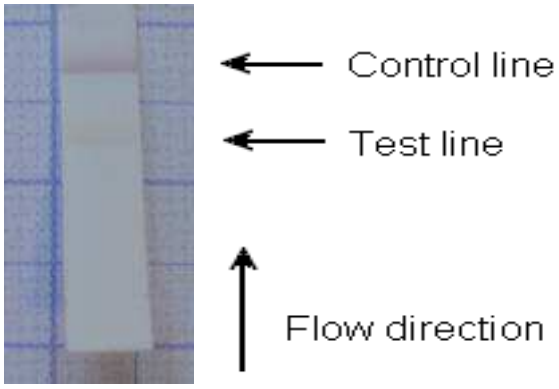

Figure 11 Standard positive test (50 µL of running buffer, 10 µL of rabbit anti mouse-colloidal gold, and 3 µL of mouse anti-TNT antibody in PBS 1X 1 µL of standard solution of TNT 0.1 mg mL^{-1})

3.2.2 Testing real samples

3.2.2.1 Dilution 1:10 in methanol

Real samples were collected from four different areas of the shelf surface by cotton swab wetted with methanol (**Figure 9**). Two different samples were collected from each area. Subsample A2 was used from area labelled A, from area labeled B subsample B1, from area labeled C subsample C1 and from area labeled D subsample D1. Each of these subsamples were diluted 1:10 in methanol and 1 µL of this extract was added to the solution described in the part about blank test, specifically 50 µL of running buffer (PBS 1X containing 1% of BSA), 10 µL of rabbit anti mouse-colloidal gold and 3 µL of mouse anti-TNT antibody in PBS 1X (1:100, v/v). As usual the reaction format is indirectly competitive: the decrease of the test line color intensity is directly correlated to the increase of the amount of TNT in the sample. **Figure 12** shows results from LFIA tests with subsamples A2, B1, C1 and D1. Due to the comparison with the blank test it is possible to say that concentrations of TNT in subsamples A2, B1 and D1 were under the LOD and so the test provided a negative result (two visible lines). In the case of subsample C1 a positive result was obtained (decrease of the color development of the test line).

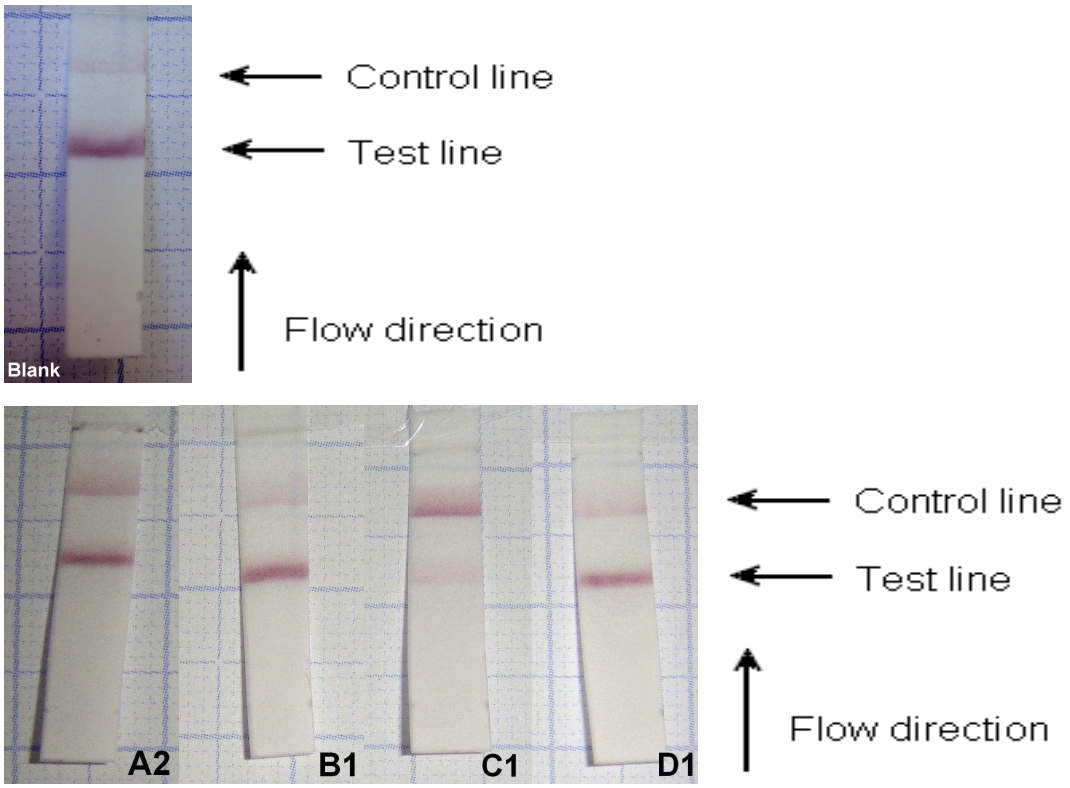

Figure 12 The blank test and results of the LFIA tests with subsamples A2, B1, C1 and D1 diluted 1:10 in MeOH

3.2.2.2 Dilution 1:100 in methanol

According to the negative results in the cases of subsamples A2, B1 and D1 in dilution 1:10 in methanol, the decision to test only subsample C1 in this dilution was made. 1 µL of subsample C1 diluted 1:100 in methanol was added to the well containing 50 µL of running buffer (PBS 1X containing 1% of BSA), 10 µL of rabbit anti mouse-colloidal gold and 3 µL of mouse anti TNT antibody in PBS 1X (1:100, v/v). Interactions between reagents are described above. The result from the LFIA test with subsample C1 is shown on **Figure 13**. This test showed less visible test line and more visible control line in comparison with the blank test, so it provided a positive result.

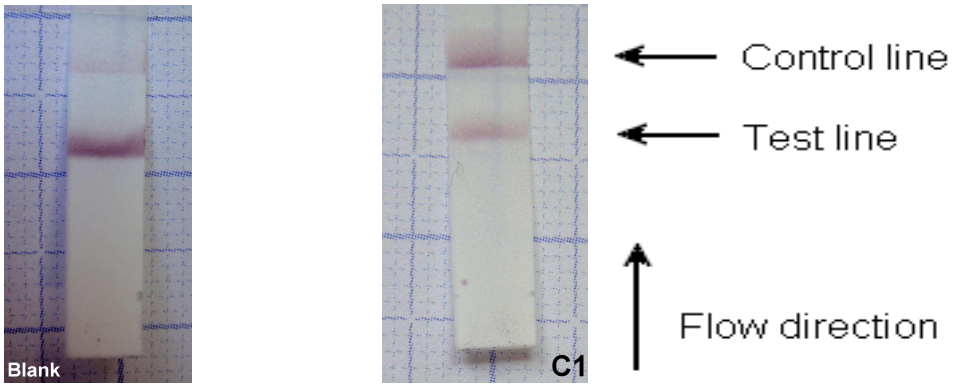

Figure 13 The blank test and result of the LFIA test with subsample C1 diluted 1:100 in MeOH

3.2.2.3 Basic concentrations of subsamples

LFIA test with subsamples A2, B1 and D1 in dilution 1:10 in MeOH gave negative results whereas subsample C1 diluted 1:10 in MeOH positive. **Figure 14** shows the results from an LFIA test performed by dipping each strip in the well containing 50 µL of running buffer (PBS 1X containing 1% of BSA), 10 µL of rabbit anti mouse-colloidal gold, 3 µL of mouse anti-TNT antibody in PBS 1X (1:100, v/v) and 1 µL of each subsample in its basic concentration. Interactions between reagents are described above. All of the subsamples provided positive results according to the comparison with the blank test. It is possible to say that subsample C1 had the highest concentration of TNT in comparison with the other tested subsamples. In this point of view subsample A2 contained less amount of TNT than subsample C1 whereas higher amount of TNT than subsamples B1 and D1.

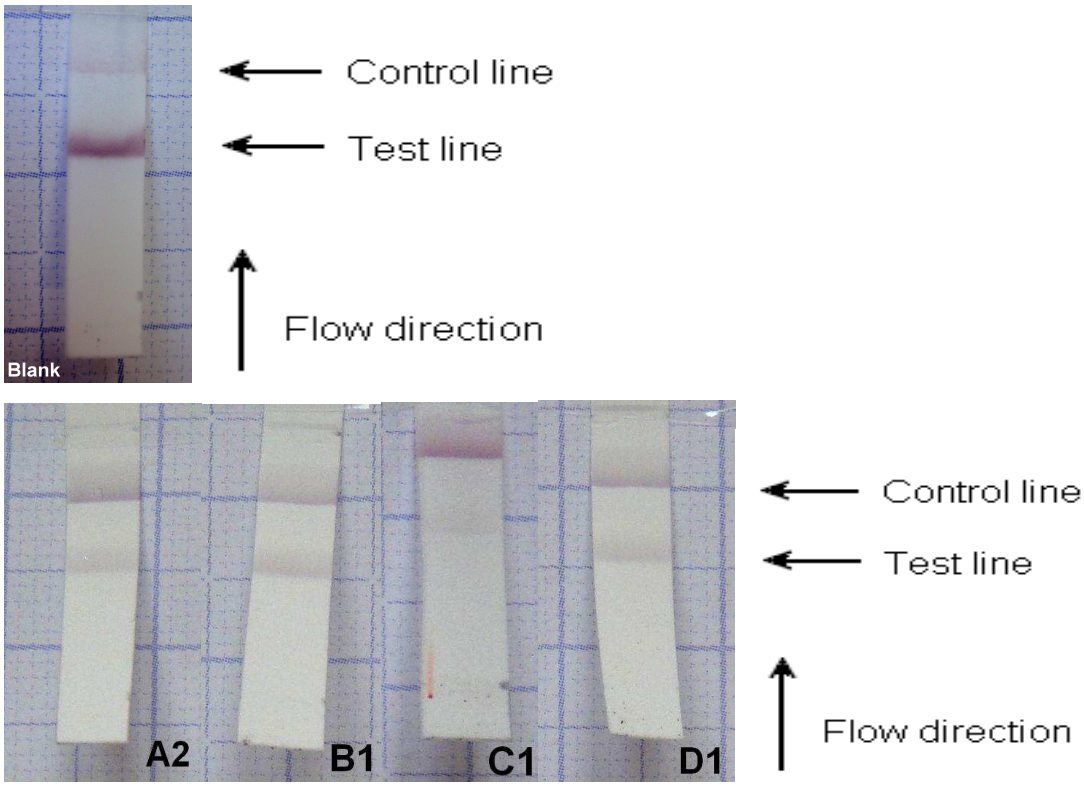

Figure 14 The blank test and results of the LFIA tests with subsamples A2, B1, C1 and D1 in their basic concentrations

3.2.3 Evaluation of the results

The summary of the results obtained during this research is presented in **Table 3**. The fact that the competitive format of the assay was used is important for evaluating the results. In the competitive format of the assay the decrease of the test line is directly correlated to the increase of the amount of TNT in the sample. Creation of the control line is a visible confirmation of proper flow throughout the LFIA strip. If the control line is not present the test is considered to be invalid. Thus only one visible (control) line gives positive result whereas two clearly visible lines (test and control lines) give a negative result. The result of each test has to be compared with the blank test, the test line of each test in confrontation with the test line on the blank test and control line of each test in confrontation with the control line of the blank test.

	Test line	Control line	Result
Blank	clearly visible	visible	-
Standard positive test	invisible	visible	+
A2	decrease in colour	visible	+
B1	decrease in colour	visible	+
C1	invisible	clearly visible	+
D1	decrease in colour	visible	+
A2 diluted 1:10	clearly visible	visible	-
B1 diluted 1:10	clearly visible	visible	-
C1 diluted 1:10	invisible	clearly visible	+
D1 diluted 1:10	clearly visible	visible	-
C1 diluted 1:100	decrease in colour	clearly visible	+

Table 3 Summary of the results

3.2.4 Comparison with chemiluminescent ELISA

As mentioned above all of the samples were also tested by chemiluminescent ELISA. This method confirmed that subsample C1 was about two orders of magnitude higher in concentration of TNT than in other subsamples. Subsample B1 contained the lowest amount of TNT. Subsamples A2 and D1 contained almost the same amount of TNT, subsample A2 a little bit more than subsample D1. ELISA is a quantitative method that can provide precise values of concentration of TNT in the sample whereas LFIA is qualitative method that can give a fast yes/no response about the presence or absence of TNT in the sample.

4. Conclusions

The lateral flow immunoassay for detection of TNT in real samples has gone through development. This method is suitable to be used anywhere where it is needed because it is simple to perform and does not require laboratory equipment. Only shaking in hand for 3 minutes was used for extraction of samples and the test was performed by dipping the LF strip in the well with small amount of reagents and the sample. LF test provides rapid results (within only 10 minutes) in case of both negative and positive results, detecting TNT from 1 μg mL^{-1}, the value of detection limit (LOD) of this LFIA.

All of the collected samples were tested by LFIA and by one other immunological method, ELISA. The results acquired by LFIA have been correlated with results obtained by ELISA. As was mentioned above, LFIA is a qualitative method providing rapid yes/no response, whereas ELISA is a quantitative method that can provide precise values of concentration of TNT in the sample, but to obtain results laboratory equipment and PC software are needed, moreover the test takes longer than in case of LFIA. According to this fact LFIA is very useful when rapid determination is necessary if the sample contains or does not contain TNT above the LOD concentration. Rapid but reliable results obtained outside the laboratory are very important in the field of criminology or environmental pollution. Reliability of the test shows two important parameters, sensitivity and specificity. Both of them were also determined during this research. Sensitivity can be defined by the LOD. As mentioned above the LOD was 1 μg mL^{-1}, which confirms good sensitivity for the qualitative method. Specificity was determined through the cross-reactivity. Some nitroaromatic compounds have low cross-reactivity but still relatively good specificity for on-site testing. In positive samples can later be analyzed accurate amount of TNT by ELISA or other analytical method.

When good reproducibility will be reached this LF test for TNT can be developed to commercial testing. During this research LFIA was used as a dipstick test, which means the strip was dipped into the well with a solution containing all the needed reagents and a sample extract. Plastic housing, conjugate (and sample) pad is appropriate for development for commercial use of this LF test.

In these days the requirement for a very fast gain of results is increasing. Because of TNT properties such as explosiveness and toxicity, its fast detection

is of utmost importance. LF tests can serve criminalists to reveal early warning of a terrorist attack or can be useful to prevent environmental pollution.

5. Bibliography

[1] Snellinx Z. et al., Biological Remediation of Explosives and Related Nitroaromatic Compounds, Environ Sci & Pollut Res (2002) 9:48-61

[2] Bradley M. D., 2,4,6-Trinitrotoluene (TNT) air concentrations, hemoglobin changes, and anemia cases in respirator protected TNT munitions demilitarization workers, Int Arch Occup Environ Health (2011) 84:239–250

[3] Leungsakul T., TNT and nitroaromatic compounds are chemoattractants for Burkholderia cepacia R34 and Burkholderia sp. strain DNT, Appl Microbiol Biotechnol (2005) 69:321–325

[4] Ayoub K. et al., Application of advanced oxidation processes for TNT removal: A review, Journal of Hazardous Materials (2010) 178:10–28

[5] Akhavan J., The Chemistry of Explosives. 2nd Edition. Cambridge: The Royal Society of Chemistry, 2004. 180 p., ISBN 0-85404-640-2

[6] Lee B., Jeong S. W., Effects of additives on 2,4,6-trinitrotoluene (TNT) removal and its mineralization in aqueous solution by gamma irradiation, Journal of Hazardous Materials (2009) 165:435–440

[7] Zukas J. A., Walters W. P., Explosive Effects and Applications. USA: Springer-Verlag New York, 1998. 435 p., ISBN 0-387-95558-5

[8] Ro K. S. et al., Solubility of 2,4,6-Trinitrotoluene (TNT) in Water, J. Chem. Eng. Data 1996, 41: 758-761

[9] Buttner W. J. et al., In situ detection of trinitrotoluene and other nitrated explosives in soils, Analytica Chimica Acta (1997) 341:63-71

[10] Kalderis D. et al., Interaction of soil, water and TNT during degradation of TNT on contaminated soil using subcritical water, Journal of Hazardous Materials (2008) 159:329–334

[11] Sheibani G, Naeimpoor F, Hejazi P., Statistical factor-screening and optimization in slurry phase bioremediation of 2,4,6-trinitrotoluene contaminated soil, Journal of Hazardous Materials (2011) 188:1–9

[12] Lotufo G. R., Whole-body and body-part-specific bioconcentration of explosive compounds in sheepshead minnows, Ecotoxicology and Environmental Safety (2011) 74:301–306

[13] Krämer P. M., Development of new rat monoclonal antibodies with different selectivities and sensitivities for 2,4,6-trinitrotoluene (TNT) and other nitroaromatic compounds, Anal Bioanal Chem (2005) 382: 1919–1933

[14] Bangs Laboratories, Inc. 9025 Technology Drive, Fishers, IN 46038-7034, Immunochromatographic, Lateral Flow or Strip Tests Development Ideas
[15] Shyu Rong-Hwa et al., Gold nanoparticle-based lateral flow assay for detection of staphylococcal enterotoxin B, Food Chemistry (2010) 118:462–466
[16] Posthuma-Trumpie G. A., Korf J., Amerongen A., Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey, Anal Bioanal Chem (2009) 393:569–582
[17] Wong R. C., Tse H. Y., Lateral Flow Immunoassay, Humana Press, a part of Springer Science‡Business Media, LLC 2009, ISBN: 978-1-58829-908-6
[18] Kolosova A. Y. et al., Development of a colloidal gold-based lateral-flow immunoassay for the rapid simultaneous detection of zearalenone and deoxynivalenol, Anal Bioanal Chem (2007) 389:2103–2107
[19] Kolosova A. Y. et al., Lateral-flow colloidal gold-based immunoassay for the rapid detection of deoxynivalenol with two indicator ranges, Analytica Chimica Acta (2008) 616:235–244
[20] Qian S., Bau H. H., Analysis of lateral flow biodetectors: competitive format, Analytical Biochemistry (2004) 326:211–224
[21] Xu Q. F. et al., Development of lateral flow immunoassay system based on superparamagnetic nanobeads as labels for rapid quantitative detection of cardiac troponin I, Materials Science and Engineering C (2009) 29:702–707
[22] Rapid Lateral Flow Test Strips, Considerations for product development, 2002, Millipore
[23] Wang X. et al., Enzyme-linked immunosorbent assay and colloidal gold immunoassay for ochratoxin A: investigation of analytical conditions and sample matrix on assay performance, Anal Bioanal Chem (2007) 389:903–911
[24] Hong W. et al., Development of an up-converting phosphor technology-based 10-channel laterál flow assay for profiling antibodies against Yersinia pestis, Journal of Microbiological Methods (2010) 83:133–140
[25] Anfossi L. et al., Development and application of a quantitative lateral flow immunoassay for fumonisins in maize, Analytica Chimica Acta (2010) 682:104–109
[26] Qian S., Bau H. H., A mathematical model of lateral flow bioreactions applied to sandwich assai, Analytical Biochemistry (2003) 322:89–98

[27] Gessler F. et al., Evaluation of lateral flow assays for the detection of botulinum neurotoxin type A and their application in laboratory diagnosis of botulism, Diagnostic Microbiology and Infectious Disease (2007) 57:243–249
[28] Zhang G. P. et al., Development of an immunochromatographic lateral flow test strip for detection of β-adrenergic agonist Clenbuterol residues, Journal of Immunological Methods (2006) 312:27–33
[29] Wang S. et al., Development of colloidal gold-based flow-through and lateral-flow immunoassays for the rapid detection of the insecticide carbaryl, Analytica Chimica Acta (2005) 546:161–166
[30] Wu J. X., Zhang S. E., Zhou X. P., Monoclonal antibody-based ELISA and colloidal gold-based immunochromatographic assay for streptomycin residue detection in milk and swine urine, Journal of Zhejiang University-SCIENCE B, 2010, Volume 11, Number 1, 52 – 60, ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
[31] Dictionary of Rheumatology, E, 2009, 60 – 61 (Online), http://www.springerlink.com/content/t51v1753hp680430/, 1.2011
[32] GeneWay Biotech (Online), http://www.genwaybio.com/gw_file.php?fid=6056, 1.2011
[33] Crowther J. R., The ELISA Guidebook, Methods in Molecular Biology, Volume 149, Totowa, New Yersey: Humana Press 2001, 446 p., ISBN 0-89603-728-2
[34] Girotti S. et al., Development of a chemiluminescent ELISA and a colloidal gold-based LFIA for TNT detection, Anal Bioanal Chem (2010) 396:687–695